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In re Application of: Britta HARDY et al.

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For: PEPTIDES USEFUL IN  
IMMUNOMODULATION

Atty. Docket No.: 85189-6100

**SUBMISSION OF CERTIFIED PRIORITY DOCUMENT**

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Sir:

Applicants have claimed priority of Israeli application no. IL145926 filed October 15, 2001, under 35 U.S.C. § 119. In support of this claim, a certified copy of said application is submitted herewith.

No fee or certification is believed to be due for this submission. Should any fees be required, however, please charge such fees to Winston & Strawn LLP Deposit Account No. 50-1814.

Respectfully submitted,

Date: 8/25/04

  
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145926 מספר:  
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15-10-2001 תאריך:  
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## Application For Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד – מקום ההתאגדות)  
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ers: Britta Hardy, Annat Raiter

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(בעברית)  
(Hebrew)

## PEPTIDE EPITOPES OR MIMOTOPES USEFUL IN IMMUNOMODULATION

(באנגלית)  
(English)

Whereby I pray for a patent to be granted to me in respect thereof.

מבקש בזאת פיי ינתן לי עליה פטנט

Division	* בקשה Application	* בקשה פטנט מוסף- Addition Application for Patent	* דרישה דין קדימה Priority Claim		
Applicant from	מבקש to Patent/Application	No..... מס. dated..... מיום.	מספר / סימן Number / Mark	תאריך Date	מדינת האגוד Convention Country
No..... מס. dated..... מיום.		No..... מס. dated..... מיום.			
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**אפיטופים פפטידיים או מימוטופים השימושיים  
באימונומודולציה**

**PEPTIDE EPITOPES OR MIMOTOPES USEFUL IN  
IMMUNOMODULATION**

# PEPTIDE EPITOPES OR MIMOTOPES USEFUL IN IMMUNOMODULATION

## 5    FIELD OF THE INVENTION

The present invention relates to the field of immunotherapy vaccines. Specifically, the present invention relates to the identification and use of epitopes or mimotopes recognized by the immunostimulatory monoclonal antibodies designated BAT, to  
10    peptides comprising these epitopes, to polynucleotides encoding these peptides and to pharmaceutical compositions comprising the peptides and/or polynucleotides and to their use in immunomodulation, especially in anti-cancer therapy, and/or for diagnostic purposes.

## 15    BACKGROUND OF THE INVENTION

Cancer is mainly treated by local therapy, such as surgical excision or ablation by radiation, however, alternative approaches based on gene therapy and immunotherapy have been attempted in the last years. One such approach is active immunotherapy in  
20    which a vaccine is administered for therapeutic and/or preventive purposes. This can include administration of immuno-potentiating agents as well as biological response modifiers such as interferons, interleukins etc., in order to stimulate the immune system. Identification of cell surface antigens expressed exclusively or preferentially on certain tumors allows the formation of selective treatment strategies. A successful  
25    vaccine for cancer immunotherapy requires the identification of a target antigen and the production of a cytotoxic T cell response.

An immune response is induced by the binding of an antigen to selected major histocompatibility complex (MHC) molecules. MHC molecules are classified as either Class I or Class II molecules. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses such as T lymphocytes, B lymphocytes, macrophages, etc. Class II molecules are recognized by helper T lymphocytes and induce their proliferation and amplification of the immune response to the epitope that is displayed. Class I MHC molecules are found on most nucleated cells and are recognized by cytotoxic T lymphocytes (CTLs) which destroy the antigen bearing cells. The CTL response is a major component of the immune system, active in immune surveillance and destruction of infected or malignant cells and invading organisms expressing foreign antigens on their surface. The ligand of the antigen-specific T lymphocyte receptor is a complex made up of a peptide fragment of a foreign antigen 8 to 10 amino acids in length, presented in the groove of MHC class I molecules. Unlike B cells, T cells do not recognize intact native antigen molecules. In general, cytotoxic T cell activation requires that the antigen be processed endogenously and cleaved into specific peptide fragments which are presented on the surface of antigen processing cells in association with class I MHC molecules.

Numerous disclosures exist concerning immunogenic peptides. WO 94/20127 discloses means and methods for selecting immunogenic peptides capable of specifically binding HLA-A2.1 allele and inducing T-cell activation. WO 95/19783 relates to peptides based on an epitope derived from the product of the tumor associated gene MAGE-3. WO 97/11715 discloses a peptide which mimics MUCI or other cancer peptides. WO 00/06723 discloses tumor specific antigen peptides and use thereof as anti-tumor vaccines.

Hardy and co-workers, in Hardy B., Dotan D. and Novogrodsky A., (1989) *Cell Immunol.* 118:22-29, in WO 95/20605 and in WO 00/58363 disclose the properties of a set of novel monoclonal antibodies against a membrane determinant of Daudi cells, a human B-lymphoblastoid cell line. The membrane determinant recognized by the antibody has an apparent molecular weight of 47-50 kDa, but has not yet been identified or characterized.

The novel antibody designated BAT was found to induce lymphocyte proliferation and cytolytic activity against tumor target cells. A single intravenous administration of BAT into mice bearing various tumors resulted in striking anti-tumor effects manifested by regression of tumors and prolongation of survival. BAT also induced regression of human tumor xenografts transplanted into SCID mice that were engrafted with human peripheral blood lymphocytes. The anti-tumor activity of BAT is mediated by its immune stimulatory properties as was evident from adoptive transfer experiments in which splenocytes from BAT treated mice injected to mice bearing tumors induced regression of tumors.

Several alternative methods of identifying the peptide epitopes bound by monoclonal antibodies are recognized in the art. These methods include the use of various peptide libraries as well as the use of phage display libraries. Phage display describes a selection technique in which a peptide or protein is expressed as a fusion protein with a coat protein of a bacteriophage, resulting in display of the fused protein on the surface of the virion, while the DNA encoding the fusion residues within the virion. Phage display has been used to create a physical linkage between a vast library of random peptide sequences to the DNA encoding each sequence, allowing the identification of peptide ligands for a variety of target molecules including antibodies by an in vitro selection process called biopanning.

Throughout this specification, various scientific publications are referenced. Additionally, various patent publications are cited in the specification. The disclosure of all these publications in their entireties are hereby incorporated by reference into this specification in order to more fully describe the state of the art to which this invention pertains.

### SUMMARY OF THE INVENTION

It is an object of the present invention to provide epitopes or mimotopes that are useful as immunomodulatory agents, for example, in stimulating immune responses and/or in tumor growth inhibition. It is a further object of the present invention to provide epitopes or mimotopes that are useful in treatment of autoimmune diseases and in treatment of cancer. It is yet a further object of the invention to provide a diagnostic agent and method for diagnosing cancer in a subject.

The term epitope referred to herein, relates to that part of an antigenic molecule that is recognized and bound by a T-cell receptor and/or by a B-cell receptor (i.e., a site on a large molecule against which an antibody will be produced and to which it will bind). The term is intended to include naturally occurring antigenic determinants or synthetic molecules that can mimic naturally occurring antigenic determinants. Molecules, which mimic the naturally occurring antigenic determinants, may also be referred to as mimotopes, and these terms may be used interchangeably in reference to epitopes which are not formed by a contiguous segment of the primary sequence of an antigen. The epitopes of the invention are recognized by the monoclonal antibodies BAT. There are thus provided, according to one embodiment of the invention, peptides comprising at least one epitope recognized by BAT monoclonal antibodies. In a

certain embodiment there are provided peptides comprising at least one epitope recognized by the monoclonal antibody BAT-1.

Further provided, according to another embodiment of the invention, are polynucleotides encoding epitopes recognized by BAT monoclonal antibodies, for example, epitopes recognized by the monoclonal antibody BAT-1. Also provided, are  
5 constructs comprising the polynucleotides and vectors comprising these constructs.

Further provided, according to a further embodiment of the invention, are cells presenting epitopes recognized by the BAT monoclonal antibody.

According to a particular preferred embodiment the peptide comprising at least one  
10 epitope recognized by the monoclonal antibody BAT-1 is a 12-mer peptide (denoted herein as Peptide A) comprising the sequence:

(Seq ID NO 1) Pro Arg Arg Ile Lys Pro Arg Lys Ile Met Leu Gln

According to another particular preferred embodiment the peptide comprising at least one epitope recognized by the monoclonal antibody BAT-1 is a 12-mer peptide  
15 (denoted herein as Peptide B) comprising the sequence:

(SEQ ID NO 2) Gln Arg Ile Leu Gln Gln Ile Asn Leu Pro Arg Ile

According to another particular preferred embodiment the peptide comprising at least one epitope recognized by the monoclonal antibody BAT-1 is a 12-mer peptide (denoted herein as Peptide C) comprising the sequence:

20 (SEQ ID NO 3) Asn Arg Ile Arg Thr Asn Thr Lys Leu Met Asn Ser

These peptides were shown to inhibit the binding of BAT-1 monoclonal antibody to Daudi cells and showed biological activity in vivo.

According to another preferred embodiment the polynucleotide of the invention  
25 comprises the sequence:



(SEQ ID NO 4) CCTCGACGAATAAAGCCCAGGAAGATCATGCTGCAA

According to yet another preferred embodiment the polynucleotide of the invention comprises the sequence:

(SEQ ID NO 5) CAGAGGATACTGCAGCAAATTAATCTTCCCAGGATC

According to yet another preferred embodiment the polynucleotide of the invention comprises the sequence:

(SEQ ID NO 6) AACCGAATCAGGACAAATACTAAGCTCATGAACAGC

The nomenclature used to describe peptide and/or polynucleotide compounds of the invention follows the conventional practice wherein the amino group (N-terminus) and/or the 5' are presented to the left and the carboxyl group (C-terminus) and/or 3' to the right.

It is to be understood explicitly that the scope of invention encompasses both shorter and longer peptides and/or polynucleotides, as well as peptide and/or polynucleotide analogs/with one or more amino acid or nucleic acid substitution, as well as amino acid or nucleic acid derivatives, non-natural amino or nucleic acids and synthetic amino or nucleic acids as are known in the art, with the stipulation that these modifications must preserve the biological activity of the original molecule. Specifically any active fragments of the active peptides as well as extensions, conjugates and mixtures are disclosed according to the principles of the present invention.

According to yet another embodiment of the invention there are provided pharmaceutical compositions for the treatment of autoimmune diseases and/or for the treatment of cancer and/or for anti-cancer or anti-tumor vaccination. The term "vaccine" or "vaccination" referred to in the specification relates to a modality or process that induces modulation of the immune system, for example, but not limited

to, a composition or process that induce the activation of T- lymphocytes or that induce the production of antibodies.

The composition according to one embodiment comprises at least one epitope recognized by the BAT monoclonal antibody. According to one embodiment the composition comprises at least one peptide comprising at least one epitope recognized by the BAT monoclonal antibody. According to another embodiment the composition comprises at least one polynucleotide encoding an epitope recognized by the BAT monoclonal antibody and/or at least one construct comprising the polynucleotide and/or at least one vector comprising the construct.

10 The present invention further provides a method for treating an autoimmune disease and/or cancer, the method comprising the step of administering to a patient a composition according to embodiments of the invention.

Also provided according to an embodiment of the invention is a method for the preparation of an immunotherapy vaccine. The method comprises the steps of panning  
15 a phage display peptide library using a biotinylated BAT monoclonal antibody thereby obtaining phages bound to the BAT monoclonal antibody; infecting a bacteria with the bound phages, thereby obtaining at least one phage clone; sequencing the at least one phage clone; determining the sequence of the peptide that binds the BAT monoclonal antibody; synthesizing a peptide according to the determined sequence;  
20 and combining the peptide with a suitable carrier to obtain an immunostimulatory vaccine.

It has previously been shown that BAT monoclonal antibodies are beneficial in treating a variety of tumors including but not limited to: melanoma, lung carcinoma, prostate cancer, breast cancer, lymphomas and leukemias, colon carcinoma, and  
25 fibrosarcomas. The peptides and/or polynucleotides of the present invention are useful

to elicit an immune response that will obviate the necessity to treat an individual with the antibodies themselves. Thus, the peptides and/or polynucleotides are useful to elicit antibodies that share the attribute of the previously known BAT antibodies.

In addition, the peptides of the invention are immunomodulatory. The peptides of the invention may serve as immunostimulatory agents to elicit anti-tumor activity or may serve as adjuvants for immunotherapy or as immune-stimulators against infections, including in immunization procedures. Conversely, they can serve to inhibit undesirable immune responses such as immune responses that are involved in inflammatory conditions, including but not limited to autoimmune diseases. As immunomodulators, these peptides can induce shifts in the immune system from undesirable responses to beneficial responses. Thus, for example the peptides of the invention could be used to induce shifts from T helper 1 (TH1) to T helper 2 (TH2) responses that have been postulated to be of therapeutic value for suppressing or preventing autoimmune diseases or disorders.

According to another embodiment of the invention there is provided a diagnostic agent and a method for diagnosing cancer in a subject. The diagnostic agent comprises an epitope recognized by the BAT monoclonal antibody and the method for diagnosing cancer comprises the steps of contacting a sample from a patient with an epitope recognized by the BAT monoclonal antibody; determining the extent of binding of the epitope to the sample, which will provide information regarding the occurrence of the BAT monoclonal antibody in the sample, and comparing the extent of binding of the epitope to the sample with a known control (such as a predetermined calibration scale). This will provide information regarding the occurrence of cancer in the sample as compared with the control.

## **BRIEF DESCRIPTION OF THE FIGURES**

The present invention will be understood and appreciated more fully from the following detailed description taken in conjunction with the appended figures in which

5 Figures 1 – 4 relate to a first experiment and Figures 5-6 relate to a second experiment.

Figure 1 is a graph showing the inhibition of BAT mAb binding to phage, bearing the insert of SEQ ID NO 1 (Peptide A);

Figure 2 shows the results of a flow cytometry experiment in which inhibition of BAT mAb binding on B-lymphoblastoid cells was determined;

10 Figure 3 is a graph showing tumor growth inhibition by immunization with the synthetic Peptide A;

Figure 4 is a graph showing binding of sera from Peptide A immunized mice to Peptide A coated plates;

Figure 5 depicts the nucleic acid and amino acid sequences of the peptides isolated in the  
15 second experiment; and

Figure 6 shows the binding of BAT mAb to screen selected library peptides in the second experiment.

## **DETAILED DESCRIPTION OF THE INVENTION**

The present invention relates to epitopes that are useful in modulation of immune responses, in tumor growth inhibition, in treatment of cancer and/or inflammatory disorders including autoimmune diseases and. As mentioned above, the term epitope is intended to include naturally occurring antigenic determinants or synthetic molecules that can mimic naturally occurring antigenic determinants. Molecules, which mimic the naturally occurring antigenic determinants, may also be referred to as mimotopes, and these terms may be used interchangeably in reference to epitopes which are not formed by a contiguous segment of the primary sequence of an antigen. The epitopes of the invention are recognized by the BAT monoclonal antibody such as described in the above-mentioned WO 95/20605. BAT monoclonal antibody is an immune modulator that induces tumor regression in mice bearing various murine and human tumors. Tumor regression is induced upon a single injection of the antibody 10-14 days after the tumor inoculation. The anti-tumor activity is mediated by T and NK lymphocytes. BAT binds a 47-50 kDa monomeric membrane protein present on B lymphoblastoid cell lines as well as on T cells. In the present invention a phage epitope library was employed in order to identify peptides that bind BAT monoclonal antibody and to prepare an immunotherapy modality. As will be further detailed below, a 12-mer-phage peptide library constructed using phage M13 was used. Three peptides were identified as the putative antibody binding epitope and their nucleic acid sequence was determined. The selected phages insert sequences were synthesized and used for inhibition of BAT binding to Daudi and Jurkat cells. Mice were immunized with the peptides in complete Freund adjuvant. Serum containing "BAT

like" antibodies bound Daudi and Jurkat cells. Tumor growth in B16 melanoma injected mice was retarded in the immunized mice compared to controls.

The peptides according to an embodiment of the invention can be isolated from natural sources, such as from Daudi cells. Alternatively, the peptides can be prepared synthetically or by recombinant DNA methods. Although the peptide will preferably be substantially free of other naturally occurring host cell proteins or protein fragments, in some embodiments the peptides can be synthetically conjugated to native fragments or particles.

The present invention includes constructs comprising the polynucleotide encoding for the epitope recognized by the BAT monoclonal antibody, for example SEQ ID NOs 4, 5 and 6, as further described below. Exemplary constructs may include promoters, enhancers and/or other regulatory sequences necessary for expression, transcription and translation, as are well known in the art. The polynucleotides can be provided with appropriate linkers and can be ligated into expression vectors available in the art.

The vectors can be used to transform suitable hosts to produce the desired protein. Vectors may include restriction enzyme sites for the insertion of additional genes and/or selection markers, as well as elements necessary for propagation and maintenance of vectors within cells.

Cells presenting epitopes recognized by the monoclonal antibody BAT are also included in the present invention. For instance dendritic cells or macrophages may be useful as immunogens. While it is known that transfected dendritic cells are useful immunogens it may also be possible to load dendritic cells with the relevant epitope by allowing them to present the peptide on their surface.

The present invention further relates to pharmaceutical compositions and methods for the treatment of autoimmune diseases and/or cancer and/or for anti-cancer or anti-

tumor vaccination. A composition according to an embodiment of the invention comprises a carrier or diluent and as an active ingredient, at least one epitope recognized by the BAT monoclonal antibody. The composition may comprise a peptide comprising the epitope or a polynucleotide encoding the epitope or encoding the peptide and/or a construct comprising the polynucleotide and/or a vector comprising the construct. The composition may comprise a single peptide or polynucleotide or a range of peptides or polynucleotides, which cover different or similar sequences. In addition, or alternatively, a single polypeptide or construct may be provided with multiple epitopes. The peptide or polynucleotide may be conjugated to a matrix or to a proteinaceous carrier, for example tetanus or diphtheria toxoids or oxidized KLH, in order to stimulate T cell help, or to other immuno-potentiating agents as well as biological response modifiers such as interferons, interleukins etc., in order to stimulate the immune system. The compositions for administration to humans may further comprise adjuvants that are suitable for human use, such as alum, which is approved for human use, or sub micron emulsions that are intended for human use as disclosed for example in WO 95/11700. Appropriate ranges of ingredients for preparing compositions with or without additional carriers or adjuvants are known in the art.

The composition may be administered dose-wise to provide an optimum therapeutic response. The active ingredient may be administered by any suitable route. Depending on the route of administration the active ingredient may need to be coated or may be prepared as a dispersion and so on. Preferably, the compositions are administered parenterally, for example, intravenously, intramuscularly, subcutaneously, and intradermally. Thus, according to an embodiment of the invention the invention provides compositions for parenteral administration which comprise a solution of the

peptides according to the invention dissolved or suspended in an acceptable carrier, such as an aqueous carrier, e.g., water, buffered water, saline, glycine, hyaluronic acid etc. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, wetting agents and the like. The composition may further comprise preservatives to prevent the growth of microorganisms.

The peptides according to an embodiment of the invention may be also administered via liposomes, slow releasing particles and the like, as known in the art, so as to increase the immunogenicity of the peptides.

A composition according to an embodiment of the invention can be directly administered to an individual for immunizing the individual. Alternatively, in accordance with an embodiment of the invention, the peptides may be used to generate new antibodies with the attribute and activities of known BAT monoclonal antibodies. Ex-vivo activation of T-cells by these peptides may also elicit the desired activity of immunostimulation. Thus, the composition can be used for inducing antibodies in an ex-vivo system and the induced antibodies can then be administered to a individual for treating an autoimmune disease, an infection or cancer. The composition can also be used in an ex-vivo system to stimulate T-cells to be administered in a process of adoptive immunotherapy, as described in the art.

The present invention also includes the use of the peptide or polynucleotide as a diagnostic agent for diagnosing cancer in an individual. According to an embodiment of the invention a sample from a individual, such as a blood sample or a sample from a patient's GI tract fluids, or cerebrospinal fluid or any other relevant sample can be contacted, in vivo or in vitro, with a peptide or polynucleotide according to the invention and the extent of binding of the peptide or polynucleotide to the sample can



be determined, such as by ELISA, so as to provide information regarding the occurrence of BAT in the sample. For example antibodies of the peptides of the invention may be used to diagnose cancer or to monitor its progression if present in a body fluid especially serum or plasma. Conversely, in the patient's lymph nodes especially in draining lymph nodes in proximity to a suspected tumor, it may be possible to screen the T cells using the peptides of the invention.

The present invention will be further described and exemplified by the following examples.

## 10 **EXAMPLES**

### **Experiment 1**

#### **Preparation, purification and biotinylation of BAT monoclonal antibody:**

BAT was generated and purified as previously disclosed in WO 95/20605, and subsequent publications. In brief, BALB-C mice were immunized with membranes of Daudi cells. Spleen cells were fused with myeloma NS-O cells. Clones producing BAT were selected by the ability of supernatants to bind Daudi cells and to induce proliferation of peripheral blood mononuclear cells. Hybridoma cells were grown in RPMI 1640 supplemented with fetal calf serum 10%, sodium pyruvate, glutamine, and antibiotics and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. BAT was purified on a protein G Sepharose column according to manufacturer's instructions (Pharmacia). Biotinylation, was performed as described: 100 µg of the immunoglobulin fraction of BAT monoclonal antibody in 1ml of 0.1 M NaHCO<sub>3</sub>, pH 8.6, was incubated for 2 hours at room temperature with 5 µg of biotin amidocaproate N-hydroxysuccinimide ester (SIGMA) from a stock solution of 1mg/ml in

dimethylformamide and dialyzed at 4°C against phosphate buffered saline pH 7.4.

#### **Isolation of Epitope-Presenting Phage from a Phage Epitope library:**

The Phage Display Peptide Library is based on a combinatorial library of random 12-mer peptides fused to a minor coat protein (pIII) of M13 phage. A library sample containing  $4 \times 10^{10}$  infectious phage particles was subjected to 6 rounds of panning and amplification. For each selection cycle 100µg of biotinylated BAT monoclonal antibody was used. The phage was pre-incubated with the biotinylated antibody at room temperature for 1 hour. The reaction mixtures were then layered in 1 ml of TBS 0.5% Tween on streptavidin coated and blocked 60-mm polystyrene Petri dishes for 30 minutes at room temperature. Unbound phages were removed by 10 times washings in TBS 0.5% Tween. The remaining phages were eluted with 1ml 0.2M Glycine -HCl (pH2.2), 1mg/ml BSA. The eluate was neutralized and used to infect E. coli stain ER2537. After each round of panning phage was titer on LB/IPTG/Xgal plates. The unamplified last round was titered and plaques were used for sequencing.

#### **Phage-ELISA:**

Wells of microtiter plates were coated with 100 µl of a 1:1000 dilution (0.1M NaHCO<sub>3</sub>, pH 8.6) of rabbit anti-phage M13 serum by incubation overnight at 4°C. Coated plates were washed 3 times with PBS 0.05% Tween 20 and 100µl of enriched phage clones, containing  $10^9$  phage particles, were then added to the wells and incubated for 1 hour at 37°C. Wells were blocked with 1% BSA in PBS for 1 hour at room temperature, washed and incubated with the antibody overnight at 4°C. For inhibition experiments, peptides were pre-

incubated with the antibody for 30 minutes, before their addition to phage coated wells. After washing, bound antibody was detected by incubation with anti-mouse IgG Peroxidase conjugated (Fab specific) for 45 minutes. After washing OPD substrate was added and the color developed was determined by an ELISA reader at 450nm.

#### **FACS analysis of Peptide inhibition experiments:**

BAT mAb biotinylated (20 $\mu$ g/ml) was incubated with different concentration of the Peptide A (0.01–40 $\mu$ g/ml) overnight at 4°C. Daudi cells ( $0.5 \times 10^6$ ) were incubated with the antibody or either with the combination of antibody and peptide for 2 hours on ice. After washing streptavidin FITC was added for 30 minutes. Cells were analyzed by a FACScan (Becton Dickinson).

#### **In vivo effect of the Peptide:**

C57BL mice were injected with 20 $\mu$ g of peptide in CFA into the foot pads. Control mice were injected only with (complete Freund's adjuvant) CFA. After seven days a boost was given with 20 $\mu$ g of Peptide A in PBS. Mice were injected with B16 ( $0.5 \times 10^6$  cells) s.c. On day 1 or on day 8 after peptide injection and volume of tumor was measured every two days. Blood was taken from mice on day 14, 21 and 28 after peptide injection for testing specific anti-peptide antibody in sera using ELISA method.

## **Results**

#### **Isolation of Phages Displaying Specificity for BAT mAb:**

In view of the anti-tumor effect of BAT mAb, we aimed to select a peptide that binds specifically the antibody from a 12-mer random phage epitope library. After each panning, enrichment in the number of plaque forming units (pfu) was observed. Resulting from 4<sup>th</sup> panning a number of  $2 \times 10^4$  pfu was obtained

and from the 6<sup>th</sup> one,  $1.5 \times 10^5$  pfu. The number of phages used in both panning was  $1 \times 10^9$ . After the sixth panning, 100% of the phages were positive for binding to BAT mAb. DNA from 40 positive clones was sequenced. The 40 phage clones exhibited the amino acid sequence: PRRIKPRKIMLQ (Peptide

5 A) and the nucleic acid sequence  
CCTCGACGAATAAAGCCCAGGAAGATCATGCTGCAA.

Binding of the phage was highly specific, because they did not bind to any control antibodies including nonrelated antibody like IgG-3 mAb.

#### 10 **Inhibition of BAT mAb binding to the selected Phages by the synthetic peptide:**

To ensure that the interaction between the selected phages and BAT mAb is caused by the insert sequences, peptides encompassing this region were synthesized. The ability of the Peptide A to compete with BAT mAb binding to  
15 the phage was assessed. The Peptide A inhibited the binding of the antibody in a dose dependent manner. Immobilized Phage on microtiter ELISA plates coated with rabbit anti-phage M13 serum was incubated with  $6 \mu\text{g/ml}$  of the antibody. The BAT mAb was pre-incubated with increased amount of synthetic Peptide A. Results are represented in absorbance 450nm in Figure 1. Figure 1  
20 shows that the synthetic peptide (Peptide A) inhibited BAT mAb binding to the phage with an inhibition concentration ( $\text{IC}_{50}$ ) value of  $6.6 \times 10^{-7}$  M when the antibody concentration was  $6 \mu\text{g/ml}$ .

**FACS analysis of Peptide inhibition experiments:**

Because BAT mAb was raised against 47-50-kDa-membrane determinant of Daudi cells we wanted to test the Peptide inhibition of the binding of this antibody to Daudi cells.

- 5 To determine whether the library-derived synthetic Peptide inhibits binding of BAT mAb to Daudi cells FACS analysis of binding percent was done. Biotinylated BAT (20µg/ml) was pre-incubated with increased amounts of the synthetic Peptide A. The mixture was added to Daudi cells and antibody bound was stained with strept-avidin FITC. Figure 2 shows different concentrations of the synthetic Peptide A (1 to 10 40µg/ml) which inhibited the binding of BAT mAb to Daudi cells in a dose dependent manner. Isotype control is shown.

**In vivo effect of the Peptide epitope:**

- Once the synthetic Peptide A was shown to interact with BAT mAb it was tested whether this peptide upon injection into mice will induce an anti-tumor effect as a result of producing anti-Peptide A antibodies in mice. C57BL mice were injected with 15 20µg of the Peptide and B16 melanoma s.c. (as described) and tumor volume was measured during 18 days. Mice were immunized with 20µg of Peptide and B16 melanoma was injected s.c. Tumor growth from 4 mice was measured every two days and tumor size was calculated. Control animals were injected only with CFA. In 20 Figure 3 the average from results of 4 mice is shown. It can be seen that mice that were immunized with Peptide A have reduced volume of tumor in comparison to the control mice that received only CFA.

- Mice were bled on day 21 and 28 after immunization. Pool of sera from 4 control mice and 4 immunized mice were incubated on microtiter ELISA plates with immobilized 25 peptide. Results are represented in absorbance 450nm in Figure 4. Figure 4 shows

using an ELISA method, that the anti-Peptide A antibodies in sera of mice that were injected with the synthetic Peptide A is higher than in controls. These results may explain the tumor growth inhibition in the treated mice.

## 5 Experiment 2

### **Preparation, purification and biotinylation of BAT monoclonal antibody:**

BAT was generated and purified as previously described. In brief, 'Balb-C mice were immunized with membranes of Daudi cells. Spleen cells were fused with myeloma NS-O cells. Clones producing BAT selected by the ability of supernatants to bind  
 10 Daudi cells and to induce proliferation of peripheral blood mononuclear cells. Hybridoma cells were grown in PFHM serum free protein media (GIBCO) and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. BAT was purified on a protein G sepharose column according to manufacturer's instructions (Pharmacia). Biotinylation, was performed as described: 100 µg of the  
 15 immunoglobulin fraction of BAT monoclonal antibody in 1ml of 0.1 M NaHCO<sub>3</sub>, pH 8.6, was incubated for 2 hours at room temperature with 5 µg of biotin amidocaproate N -hydroxysuccinimide ester (SIGMA) from a stock solution of 1mg/ml in dimethylformamide and dialyzed at 4°C against phosphate buffered saline pH 7.4.

### **Isolation of Epitope-Presenting Phage from the Phage Epitope library:**

20 The Phage Display Peptide Library is based on a combinatorial library of random peptide 12-mer fused to a minor coat protein (pIII) of M13 phage. A library sample containing  $2 \times 10^{10}$  infectious phage particles was subjected to 3 rounds of panning. For biopanning selection cycles, 20µg of biotinylated BAT monoclonal antibody was used. The phage was pre-incubated with the biotinylated antibody at room temperature for 1  
 25 hour. The reaction mixture was then layered in 1 ml of TBS 0.5% Tween on strept-

avidin coated and blocked 60-mm polystyrene Petri dishes for 30 minutes at room temperature. Unbound phages were removed by 10 times washings in TBS 0.5% tween. The remaining phages were eluted with 1ml 0.2M Glycine -HCl (pH2.2), 1mg/ml BSA. The eluate was neutralized and used to infect E.coli strain ER2537.

5 After each round of panning phage was titer on LB/IPTG/Xgal plates. After the second cycle of biopanning, the number of phages was amplified. The an-amplified last round was tittered and plaques were used for DNA sequencing.

#### **Phage-ELISA:**

Wells of microtiter plates were coated with 100  $\mu$ l of a 1:1000 dilution (0.1M NaHCO<sub>3</sub>, pH 8.6) of rabbit anti-phage M13 serum by incubation overnight at 4°C.

Coated plates were washed 3 times with PBS 0.05% Tween. Then, 100 $\mu$ l of enriched phage clones, containing 10<sup>9</sup> phage particles, were then added to the wells and incubated for 1 hour at 37°C. After incubation, wells were blocked with 1% BSA in PBS for 1 hour at room temperature, washed and incubated with the antibody overnight at 4°C. After washing bound antibody was detected with IgG Peroxidase conjugated (Fab specific) for 45 minutes. After washing OPD subtract was added and the color developed was determined by an ELISA reader at 450nm.

#### **Results**

##### **Isolation of Phages Displaying Specificity for BAT mAb:**

20 In view of the anti-tumor effect of BAT mAb, it was aimed to select a peptide that binds specifically the antibody from a 12-mer random phage epitope library. After each panning enrichment in the number of plaque forming units (pfu) was observed. Resulting from 1<sup>st</sup> panning a number of 5.6x10<sup>6</sup> pfu was obtained, from the 2<sup>nd</sup> one, 2x10<sup>2</sup> pfu, after amplification the number of pfu was 2x10<sup>8</sup> and after the last round of  
25 biopanning 1x10<sup>3</sup> pfu were counted (Table 1).

5

**Table 1: Screening for BAT binding peptide.**

	Panning1	Panning2	Panning3
Number of phages:	$2 \times 10^{10}$	$5.6 \times 10^6$	$2 \times 10^8$
PFU obtained	: $5.6 \times 10^6$	$2 \times 10^2$	$2 \times 10^3$ (sequenced 52)
Percent	: 0.028%	0.004%	0.0005%

10

15 DNA from 42 positive clones was sequenced. The DNA from 31 of the phage clones exhibited the sequence:

CAGAGGATACTGCAGCAAATTAATCTTCCCAGGATC (Peptide B),

3 of them exhibited the sequence:

AACCGAATCAGGACAAATACTAAGCTCATGAACAGC (Peptide C)

20 and the rest of the positive clones exhibited different sequences (Table 2 and Figure 5).

**Table 2: sequencing results:**

Peptide B 31/52 identical insert

Peptide C 3/52 identical insert

Others: N 8/52 different inserts

25



**Phage ELISA on anti-M13 plates:**

To ensure that the interaction between the selected phages and BAT mAb is caused by the insert sequences, ELISA on anti M13 binding plates was performed. The ability of the positive phage to bind BAT mAb was assessed.  $1 \times 10^9$  phages from positive pfu  
5 were incubated on anti-M13 plates and binding to BAT mAb was assessed. ELISA detected only phages with peptide B in the insert. Phages with the peptide B sequence (51-52) bound the antibody when phages with the other sequences (40, 6, 7, and 37) did not. Figure 6 represents the binding of phages to BAT monoclonal antibody (5 $\mu$ g/ml).

10

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described herein above. Rather the scope of the invention is defined by the claims which follow:

**CLAIMS**

1. A peptide comprising at least one epitope recognized by a BAT monoclonal antibody.
- 5 2. The peptide according to claim 1 comprising 12 amino acid residues, or an active fragment thereof.
3. The peptide according to claim 1 comprising SEQ ID NO 1, or an active fragment thereof.
4. The peptide according to claim 1 comprising SEQ ID NO 2, or an active  
10 fragment thereof.
5. The peptide according to claim 1 comprising SEQ ID NO 3, or an active fragment thereof.
6. The peptide according to claim 1 comprising a sequence selected from the group consisting of: SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, active  
15 fragments thereof, or a combination thereof.
7. A peptide configured for being capable of inhibiting binding of BAT monoclonal antibody to lymphoma cells.
8. The peptide according to claim 7 wherein the lymphoma cells are Daudi or Jurkat cells.
- 20 9. The peptide according to claim 7 comprising 12 amino acid residues, or an active fragment thereof.
10. The peptide according to claim 7 comprising SEQ ID NO 1, or an active fragment thereof.
11. The peptide according to claim 7 comprising SEQ ID NO 2, or an active  
25 fragment thereof.

12. The peptide according to claim 7 comprising SEQ ID NO 3, or an active fragment thereof.
13. The peptide according to claim 7 comprising a sequence selected from the group consisting of SEQ ID NO1, SEQ ID NO. 2, SEQ ID NO 3, or active fragments thereof, or combinations thereof.
14. A peptide for inhibiting tumor growth comprising at least one epitope recognized by a BAT monoclonal antibody.
15. The peptide according to claim 14 comprising 12 amino acid residues, or an active fragment thereof.
16. The peptide according to claim 14 comprising SEQ ID NO 1 or an active fragment thereof.
17. The peptide according to claim 14 comprising SEQ ID NO 2, or an active fragment thereof.
18. The peptide according to claim 14 comprising SEQ ID NO 3, or an active fragment thereof.
19. The peptide according to claim 14 comprising a sequence selected from the group consisting of SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, or active fragments thereof, or combinations thereof.
20. A peptide configured for being capable of inducing an immune response against tumor cells comprising at least one epitope recognized by a BAT monoclonal antibody.
21. The peptide according to claim 20 comprising 12 amino acid residues or an active fragment thereof.
22. The peptide according to claim 20 comprising SEQ ID NO 1, or an active fragment thereof.

23. The peptide according to claim 20 comprising SEQ ID NO 2, or an active fragment thereof.
24. The peptide according to claim 20 comprising SEQ ID NO 3, or an active fragment thereof.
- 5 25. The peptide according to claim 20 comprising a sequence selected from the group consisting of SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, or active fragments thereof, or combinations thereof.
26. A polynucleotide encoding for at least one epitope recognized by a BAT monoclonal antibody.
- 10 27. The polynucleotide according to claim 26 comprising 36 nucleotide bases.
28. The polynucleotide according to claim 26 comprising SEQ ID NO 4 or an active fragment thereof.
29. The polynucleotide according to claim 26 comprising SEQ ID NO 5, or an active fragment thereof.
- 15 30. The polynucleotide according to claim 26 comprising SEQ ID NO 6, or an active fragment thereof.
31. The polynucleotide according to claim 26 comprising a sequence selected from the group consisting of SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 or active fragments thereof, or a combination thereof.
- 20 32. A construct comprising the polynucleotide according to any one of claims 26 – 31.
33. A vector comprising the construct according to claim 32.
34. A cell comprising a construct according to claim 32.
35. A cell comprising a vector according to claim 33.

36. A pharmaceutical composition for treating cancer comprising at least one peptide according to any of claims 1 – 25 and a suitable carrier.
37. A pharmaceutical composition for treating cancer comprising at least one polynucleotide according to any one of claims 26 – 32 and a suitable carrier.
- 5 38. A pharmaceutical composition for treating cancer comprising a construct according to claim 32 and a suitable carrier.
39. A pharmaceutical composition for treating cancer comprising at least one cell according to claim 34 and a suitable carrier.
- 10 40. A method for treating cancer comprising the step of administering to a subject in need thereof at least one dose of the composition according to any of claims 37 – 39.
41. A method for the preparation of an immunostimulatory vaccine comprising the steps of:
- 15       panning a phage display peptide library using a biotinylated immunostimulatory monoclonal antibody thereby obtaining phages bound to the immunostimulatory monoclonal antibody;
- infecting a bacteria with the bound phages, thereby obtaining at least one phage clone;
- sequencing the at least one phage clone;
- 20       determining the sequence of the insert peptide;
- synthesizing a peptide according to the determined sequence of the insert peptide; and
- combining the synthesized peptide with a suitable carrier to obtain an immunostimulatory vaccine.

42. The method according to claim 41 wherein the immunostimulatory monoclonal antibody is a BAT monoclonal antibody.

43. A diagnostic agent for detecting the presence of tumor cells comprising the peptide according to any of claims 1 – 25.

5 44. A diagnostic agent for detecting the presence of tumor cells comprising the polynucleotide according to any of claims 26 - 32.

45. A method for diagnosing cancer comprising the steps of:

contacting a sample from a subject with an epitope recognized by a BAT monoclonal antibody;

10 determining the extent of binding of the epitope to the sample; and

comparing the extent of binding of the epitope to the sample with a known control, thereby obtaining information regarding the occurrence of cancer in the sample.

46. A method for diagnosing cancer comprising the steps of:

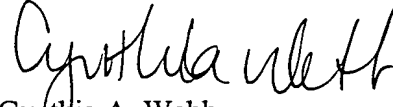
15 contacting a sample from a subject with an epitope recognized by a BAT monoclonal antibody;

determining the extent of binding of the epitope to the sample; and

20 comparing the extent of binding of the epitope to the sample with a known control, thereby obtaining information regarding the occurrence of cancer in the sample.

47. A method for diagnosing an inflammatory disorder or autoimmune disease  
comprising the steps of:  
obtaining a sample of immune cells from an individual;  
exposing the cells to an epitope recognized by a BAT monoclonal antibody;  
5 and monitoring the response of immune cells.

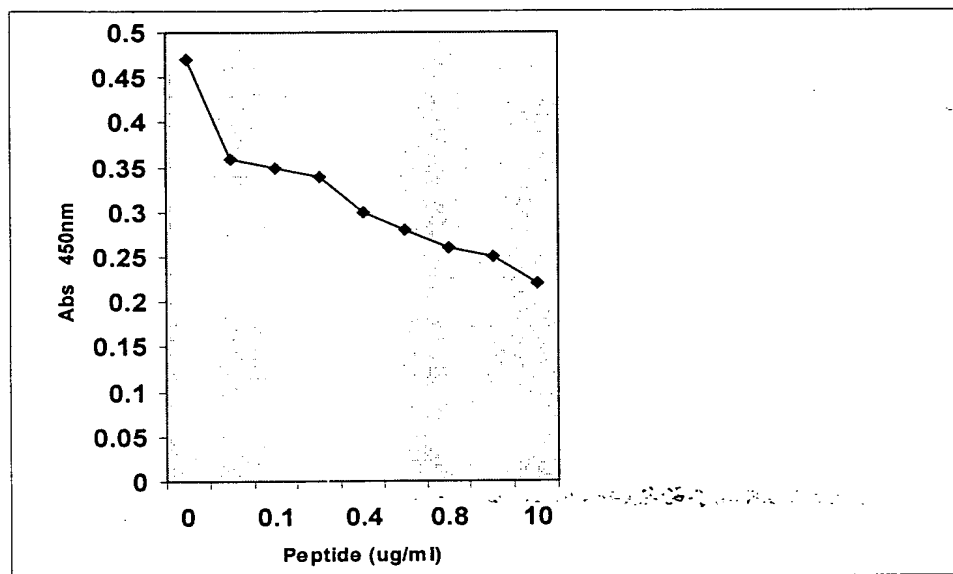
For the Applicant,



Cynthia A. Webb,  
Webb, Ben-Ami & Associates

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FIGURE 1





**FIGURE 2**

Peptide inhibition of BAT binding to Daudi cells; FACS analysis.

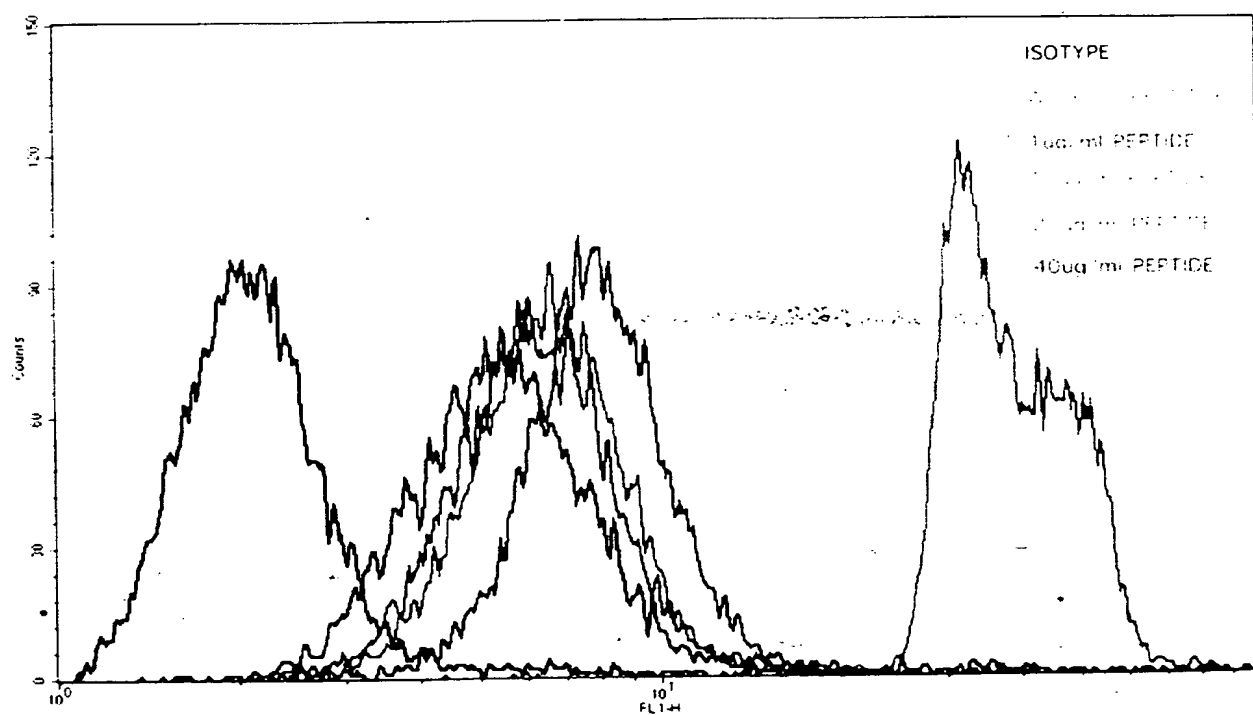


FIGURE 3

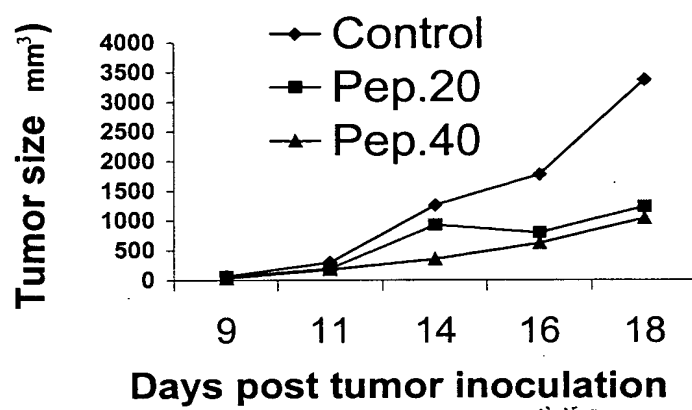
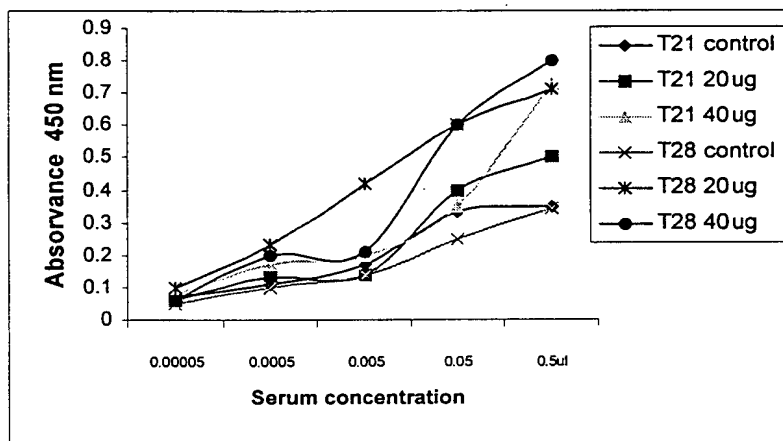


FIGURE 4



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**FIGURE 5**

**Peptide B**

**DNA** CAGAGGATACTGCAGCAAATTAATCTTCCCAGGATC

**Protein** gln arg ile leu gln gln ile asn leu prol arg ile

**Peptide C**

**DNA** AACCGAATCAGGACAAATACTAAGCTCATGAACAGC

**Protein** asn arg ile arg thr asn thr lys leu met asn ser

**Peptide N**

**DNA** ATGAGGAGCCACCCGAAGAACCGGAGAAATCACAGA

**Protein** met arg ser his prol lys asn arg arg asn his arg

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FIGURE 6

